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3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	UNILEVER PLC UNILEVER HOUSE BLACKFRIARS LONDON EC4P 4BQ GB 0317985.0
	00001628002 Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	GB
4.	Title of the invention	PLANT AND PLANT EXTRACTS AND THEIR USE
5.	Name of your agent *(if you have one)*	LLOYD WISE
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	COMMONWEALTH HOUSE 1-19 NEW OXFORD STREET LONDON WC1A 1LW ENGLAND
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
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11. I/We request the grant of a patent on the basis of this application.

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LLOYD WISE

Date

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ANNA HALLY
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PLANT AND PLANT EXTRACTS AND THEIR USE

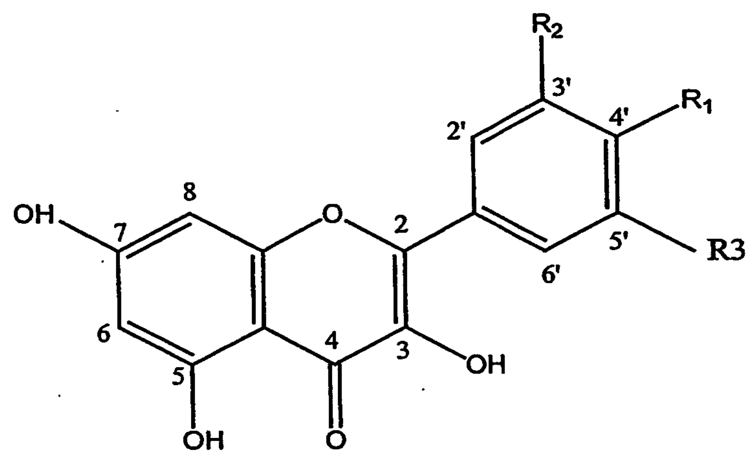
FIELD OF THE INVENTION

The present invention relates to use of a plant or plant extract, preferably a vegetable or fruit, which exhibits naturally high, increased or altered levels of flavonol glycosides in the reduction of hypertension in mammals, such as man, and novel products containing those flavonol glycosides.

BACKGROUND OF THE INVENTION

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin and are well known for their antioxidant capacities. Major dietary sources of flavonoids are vegetables, fruits, and beverages such as tea and red wine. Among the dietary flavonoids, quercetin-glycosides are amongst the most abundant. Flavonoids in general have been reported to confer a number of health benefits and are believed to act by intervention in various metabolic pathways such as by inhibition of 5-cyclooxygenase. Included within the general term flavonoid are flavonols, flavones, flavanones, catechins, anthocyanins, isoflavonoids, dihydroflavonols and stilbenes.

The main types of flavonols found in plants are based on quercetin, kaempferol and myrecetin, and their respective glycosides.



Kaempferol: R1 = OH, R2=H, R3=H
Quercetin: R1 = OH, R2=OH, R3=H
Myrecetin: R1 = OH, R2=OH, R3=OH

This figure depicts the three different flavonol aglycones (no sugars attached). The sugars are usually attached to the 3 and 7 positions, but attachments on the 4' and 3' and possibly even 5 positions feature as well. The sugars are either attached as monomers, dimers and sometimes trimers. More than one attachment site can be used, although 4 sugars appears to be the maximum number observed. Flavonols represent a large class of molecules all based on a small number of core structures and natural variation is achieved by attachment of other molecular entities e.g. sugar, methyl groups etc, at different positions of the flavonol core-ring structure. Glycosylated forms are very

abundantly found in nature, although the un-glycosylated form (aglycon) can occur as well.

Different plants have different profiles of flavonol glycosides. For example, onions are rich in quercetin-3,4'-diglucoside and quercetin-4'-glucoside. In addition, they contain smaller amounts of 3-glucoside, 4',7-diglucoside and of rutin (3-rutinoside). Apples contain rutin, quercetin-3-galactoside, quercetin-3-arabinofuranoside, quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin-3-xyloside, quercetin-3-arabinoside. Tea contains rutin as the main flavonol, but also contains quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-rhamnoside-diglucoside. Buckwheat contains high levels of rutin in the leaves and flowers and is the main commercial source for rutin supplements on the market. Tomato contains rutin as the main flavonol. Broccoli and kale are good sources of quercetin-glycosides and contain even more kaempferol-glycosides (about twice the amount of quercetin-glycosides). Kaempferol-glycosides are routinely found in many plants alongside quercetin-glycosides but often, although not always, in much smaller quantities.

Onions, mainly yellow and red onions, are the food crops with the highest natural levels of quercetin-glycosides and typically contain about 300-600 mg/kg fresh weight (FW) of flavonols. Similar, albeit slightly lower levels are present in berries such as cranberries, lingonberries, bilberries and

blackcurrants. Other major sources are apples which can have up to 100mg/kg FW and tea which can have about 25mg per cup of tea. Tomatoes when unmodified typically contain about 10 to 20 mg flavonols per kg FW, prototype high flavonol tomato varieties have been shown to contain 350 mg/kg FW, whilst concentrated tomato paste made from such prototypes contains about 1200 mg/kg FW.

In unmodified tomato fruits, the main flavonoid found is naringenin chalcone (Hunt et al, Phytochemistry, 19, (1980), 1415-1419). It is known to accumulate almost exclusively in the peel and is simultaneously formed with colouring of the fruit. In addition to naringenin chalcone, glycosides of quercetin and, to a lesser extent, kaempferol are also found in tomato peel.

Verhoeyen M. et al "Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway" J Exp Botany (2002) 377: 2099-2106, outlines the various approaches to enhance flavonoid biosynthesis in tomatoes. Methods for increasing the production of flavonoids in plants by manipulating gene activity in the flavonoid biosynthetic pathway are disclosed in WO-A-99/37794, WO-A-00/04175 and EP 1254960.

An elevated blood pressure or hypertension has a prevalence of about 15 % in Western populations and is increasing in developing countries. Above the age of 65 the incidence increases to approximately 35%. Hypertension is an

established and independent risk factor for coronary heart disease (CHD), kidney and heart failure and stroke and may lead to disability and premature death. Lowering blood pressure in hypertensive subjects is effective in reducing the risk and disability of associated diseases. Specifically, published epidemiological studies have shown that lowering blood pressure in humans by even a few mmHg reduces the incidence of several cardiovascular diseases. For example, lowering systolic blood pressure by 5 mmHg reduces all-cause mortality by 7% on a population basis, while coronary heart disease and stroke was reduced by 9 and 14%, respectively (Whelton et al. (2002) JAMA 288:1882-1888).

Spontaneously Hypertensive Rats (SHR) are considered to be a representative model of human essential hypertension. These rats are generally used to understand the development and establishment of hypertension and to determine the blood pressure lowering effect of newly synthesised anti-hypertensive drugs. In a recent study by Duarte et al., "Effects of chronic quercetin treatment on hepatic oxidative status of spontaneously hypertensive rats" Mol. Cell Biochem (2001) 221:155-160, it was shown that SHR are characterised by increased hepatic and plasma malondialdehyde concentrations, indicating increased oxidative stress. Duarte's group further found that treatment of SHR with quercetin aglycone reduced blood pressure, increased glutathione peroxidase activity and reduced both plasma and hepatic malondialdehyde levels. It was concluded

that quercetin aglycone therefore shows both antihypertensive and antioxidant properties in this model of genetic hypertension (SHR).

We have now found that plant or plant extracts, preferably tomatoes or tomato extracts, which are enriched in glycosylated forms of quercetin can demonstrably lower blood pressure in a mammal such as SHR. The finding that foods enriched in such a substance, which occurs naturally and can be incorporated as part of a regular diet can lower blood pressure is significant.

DEFINITION OF THE INVENTION

A first aspect of the present invention provides the use of a plant or plant extract which exhibits naturally high, increased or altered levels of flavonol glycosides in the reduction of hypertension. Preferably, the plant is genetically modified to exhibit increased levels of flavonol glycosides. The plant is preferably a fruit or vegetable.

According to a second aspect there is provided a food product or health supplement containing a plant or plant extract, preferably a fruit or vegetable or fruit or vegetable extract, which exhibits naturally high, increased or altered levels of flavonol glycosides.

According to a third aspect there is provided a method for the treatment of hypertension in mammals by administering to the mammal an effective

amount of a plant or plant extract, preferably a fruit or vegetable, which may be genetically modified, which exhibits naturally high, increased or altered levels of flavonol glycosides or a food product or health supplement containing the plant or plant extract, most preferably a fruit or vegetable or fruit or vegetable extract, in the reduction of hypertension.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 a and b show the change in average diastolic blood pressure of SHR relative to the average diastolic blood pressure in week 0, during the period from 9am to 7am.

1a. Change in average diastolic blood pressure relative to average diastolic blood pressure in week 0 is shown for each diet group for week 0 and for the 5 weeks of experimental food.

1b. Change in average diastolic blood pressure relative to average diastolic blood pressure in week 0 is shown for each diet group for week 5 of experimental food.

Figures 2a and b show the change in average systolic blood pressure of SHR relative to average systolic blood pressure in week 0, during the period from 9am to 7am

2a. Change in average systolic blood pressure relative to average systolic blood pressure in week 0 is shown for each diet group for week 0 and for the 5 weeks of experimental food.

2b. Change in average systolic blood pressure relative to average systolic blood pressure in week 0 is shown for each diet group for week 5 of experimental food.

Figures 3 a and b shows the change in average mean blood pressure of SHR relative to average mean blood pressure in week 0, during the period from 9am to 7am.

3a. Change in average blood pressure relative to average mean blood pressure in week 0 is shown for each diet group for week 0 and for the 5 weeks of experimental food.

3b. Change in average mean blood pressure relative to average mean blood pressure in week 0 is shown for each diet group for week 5 of experimental food.

Figures 4 a and b show the change in average diastolic pressure in SHR relative to average diastolic blood pressure in week 0, during the period from 5am to 7am (early morning period).

4a. Change in average diastolic blood pressure relative to average diastolic blood pressure in week 0 is shown for each diet group for week 0 and for the 5 weeks of experimental food.

4b. Change in average diastolic blood pressure relative to average diastolic blood pressure in week 0 is shown for each diet group for week 5 of experimental food.

Figures 5 a and b show the change in average systolic pressure in SHR relative to average systolic blood pressure in week 0, during the period from 5am to 7am (early morning period).

5a. Change in average systolic blood pressure relative to average systolic blood pressure in week 0 is shown for each diet group for week 0 and for the 5 weeks of experimental food.

5b. Change in average systolic blood pressure relative to average systolic blood pressure in week 0 is shown for each diet group for week 5 of experimental food.

Figures 6 a and b show the change in average mean pressure in SHR relative to average mean blood pressure in week 0, during the period from 5am to 7am (early morning period).

6a. Change in average mean blood pressure relative to average mean blood pressure in week 0 is shown for each diet group for week 0 and for the 5 weeks of experimental food.

6b. Change in average mean blood pressure relative to average mean blood pressure in week 0 is shown for each diet group for week 5 of experimental food.

DETAILED DESCRIPTION OF THE INVENTION

Any plant, preferably a fruit or vegetable which exhibits naturally high increased, or altered levels of flavonol glycosides, preferably quercetin glycosides, can be used to reduce hypertension in mammals. Most preferably rutin or isoquercitrin or both are used.

When the plant is used *per se* it must exhibit naturally high, increased or altered levels of flavonol glycosides. Where a plant extract is used, the level of flavonols exhibited in the plant is not as important as the extract may be manufactured to contain any desired concentration of flavonols.

Preferably, the daily dose of flavonols provided by the plant or plant extract is the amount of flavonol glycosides equivalent to from about 0.1 to 20mg of quercetin aglycon per kg of body weight (BW), more preferably from about 1 to 20 mg of quercetin aglycon per kg of BW, even more preferably from 10 to 20 mg of quercetin aglycon per kg of BW.

For example, an amount of flavonols equivalent to about 0.425 mg of quercetin aglycon per kg of BW may be used. The molecular weight (MW) of quercetin is 338.26, and the MW of isoquercitrin and rutin are 464.4 and 610.53 respectively. Thus, 0.583 mg isoquercitrin is equivalent to 0.425 mg of quercetin aglycon, whilst 0.767 mg rutin is also equivalent to 0.425 mg of the quercetin aglycon.

Preferably, the plants according to the invention are plants with a history of human consumption. Suitable plants are for example vegetables, fruits, nuts, herbs, spices, infusion materials. Suitable vegetables are for example from the Pisum family such as peas, family of Brassicae, such as green cabbage, Brussel sprouts, cauliflower, the family of Phaseolus such as barlotti beans, green beans, kidney beans, the family of Spinacea such as spinach, the family of Solanaceae such as potato and tomato, the family of Daucus, such as carrots, family of Capsicum such as green and red pepper, and berries for example from the family of Ribesiaceae, Pomaceae, Rosaceae, for example strawberries, black berries, raspberries, black currant, bilberry, lingonberry, cranberry and edible grasses from the family of Gramineae such as maize, and citrus fruit for example from the family of Rutaceae such as lemon, orange, tangerine. Also preferred are plants which can form the basis of an infusion such as black tea leaves, green tea leaves, jasmin tea leaves. Also preferred is buckwheat.

A particularly preferred plant for use in the method according to the invention is the tomato plant.

Where a plant *per se* is used, the plant may have "naturally high" levels of flavonol glycosides. "Naturally high" in this context means about 50 mg/kg FW and above. For example, onions may be used which have naturally high levels of flavonols.

An "altered" level of flavonoids is used throughout this specification to express that the level of specific flavonoids in a transformed plant differs from the level of flavonoids present in untransformed plants. Preferably, the difference is between 2 and 100 fold.

Preferably, the fruit or vegetable is genetically modified to exhibit altered levels of flavonol glycosides compared to the wild type plant. Methods for manipulating the production of flavonoids in plants by manipulating gene activity in the flavonoid biosynthetic pathway are disclosed in WO-A-99/37794, WO 00/04175, EP 1254960 and Verhoeyen M. et al "Increasing antioxidant levels in tomatoes through modification of the flavonoid biosynthetic pathway" J Exp Botany (2002) 377 : 2099-2106.

Reference to the term “increased levels of flavonol glycosides” means levels higher than normally produced in such fruit or vegetables. Preferably, the level of flavonoids is at least 4 times higher than in similar untransformed plants, more preferably from 10 to 100 times higher than in similar untransformed plants.

It will of course be understood that the plant does not have to be genetically modified to provide “increased” levels of flavonoids. Such plants develop naturally or by conventional cross-breeding.

Measuring the amount of flavonol glycosides in fruit or vegetables can be carried out using known techniques such as HPLC as shown in WO-A-99/37794 and WO 00/04175. Thus, the skilled man would be able to determine the level of flavonols in a plant and compare that to the levels normally produced in the wild type plant to determine whether flavonoid production was “altered” or “increased” or “nauturally high”, using the techniques outlined in WO-A-99/37794 and WO 00/04175.

Where a plant extract is used, the “increase” or “alteration” in the levels of flavonols of the plant being used may be minimal over the wild type plant, or the wild type plant itself may be used. When a plant extract is used, the concentration of the flavonol may be determined without reference to the plant the extract is derived from. Hence, the plant extract concentration is

determined independently to that of the plant it is derived from. The plant extract may be from a modified or unmodified plant. The important feature is that the plant extract provides the required minimum dosage of flavonols.

Modification of a plant to up-regulate flavonoid synthesis can occur using several different techniques, as follows.

For example, through the ectopic expression of either a select number of key biosynthetic genes or key regulatory elements, or a combination of both. In peel tissue, chalcone isomerase gene activity appears to be critical in WO 00/04175 and expression of a sequence encoding the *P. hybrida* chalcone isomerase has been shown to lead to a large increase in the level of quercetin-glycoside accumulation. It has further been demonstrated in EP 1254960 that concomitant expression of the sequences encoding chalcone synthase and flavonol synthase from *P. hybrida* is sufficient to achieve accumulation of kaempferol-glycosides in tomato flesh. In addition, studies have shown that ectopic expression of three genes encoding the biosynthetic enzymes CHS, CHI and FLS achieve increased flavonol accumulation throughout tomato fruit. Alternatively, ectopic expression of the regulatory genes Lc and C1, together with the biosynthetic gene CHI results in a similar phenotype.

Most preferably, the fruit is a genetically modified tomato which exhibits increased levels of flavonol glycosides, such as the tomatoes described in WO 00/04175. Specifically, tomato plants can be transformed with a sequence from *P. hybrida* encoding CHI, under the control of the strong constitutive double CaMV35S promoter. Analysis of such transformants containing the CHI transgene show a dramatic increase in fruit peel flavonol levels compared with control plants, up to 78-fold increase in individual fruits. This rise in total flavonol accumulation mainly comprised increases in the accumulation of rutin (quercetin 3-*0*-rutinoside), isoquercitrin (quercetin-3-*0*-glucoside) and kaempferol-3-*0*-rutinoside in the peel tissues.

An alternative method according to WO-A-99/37794 and Bovy et al, "High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes Lc and C1 " The Plant Cell (2001), 14, 2509-2526, which may be used involves transforming the tomato with transcription factors such as Lc and C1. In general, this method may involve the incorporation of two or more genes each encoding a different transcription factor for flavonoid biosynthesis, or a sequence functionally equivalent thereto, each being operably linked to a promoter.

In a further alternative approach, P. *hybrida* sequences encoding each of the key biosynthetic enzymes leading to flavonols, chalcone synthase (CHS), chalcone isomerase (CHI), flavonone-3-hydroxylase (F3H), and flavonol

synthase (FLS) were ectopically expressed simultaneously. HPLC analyses of primary transformants containing all four transgenes showed that these tomato lines accumulate very high levels of quercetin glycosides in the peel and, more modest, but significantly increased levels of kaempferol- and naringenin-glycosides in columella tissue (Colliver *et al.*, Phytochemistry Reviews (2002) 1: 113-123. Improving the nutritional content of tomatoes through reprogramming their flavonoid biosynthetic pathway). The high quercetin phenotype in the peel was expected because of the presence of the CHI transgene, and it is noteworthy that the levels detected were similar to those found in CHI-only transformants.

In addition to the 'single gene' transformants, 'two-gene' combinations can be used which involve crossing of parent plants harbouring single gene constructs. HPLC analyses of fruit from these transformed lines revealed that the genes that appear to be critical in leading to flavonol biosynthesis in tomato flesh (pericarp and columella) tissue are CHS and FLS. As described by Colliver et al, ectopic expression of CHS resulted in modified tomatoes accumulating increased levels of naringenin-glycosides but with no increase in flavonols. By contrast, analysis of tomatoes harbouring the FLS transgene showed that no significant difference in bio-chemical phenotype was detectable when compared to control fruit. The analyses have shown that concomitant expression of both CHS and FLS has a synergistic effect

resulting in a significant accumulation of both naringenin- and kaempferol-glycosides in tomato flesh.

The plant, preferably fruit or vegetable, exhibiting altered or increased levels of flavonoids may be administered in different forms such as in food products or health supplements. It is to be understood that the plant *per se* may be used or a plant extract with high or "naturally high" levels of flavonols may alternatively or additionally be used.

For example, once harvested the plants may be eaten as such. Alternatively, the fruit or vegetables may be used in the production of food products or health supplements. For example parts of the fruit or vegetable may be added to salads. Also, heat-treatment may be applied, for example tomatoes may be used to prepare tomato sauces with tomato as one of the main ingredients (e.g. at levels of about 10% by weight or more, for example 80% by weight or more) such as tomato paste, tomato ketchup, pizza sauce, pasta sauce, dressings etc. Also the tomatoes may be used to prepare products like tomato juice, tomato soups etc.

In addition, the food products can be selected from the group consisting of nutritional supplements, spreads, margarines, creams, sauces, dressings, mayonnaises, ice creams, fillings, confectioneries, health bars, cereals, health

drinks. In this case an extract of the fruit or vegetables or other plants such as tea, onions or buckwheat exhibiting high levels of flavonoids may be used.

In addition to the above components the blends and the food products can contain other micronutrients, examples thereof being anti oxidants (Vitamin C or Vitamin E), other vitamins in particular Vitamin B1, B6 and B12, Vitamin K, folic acid, minerals like calcium, magnesium, iron, copper, or zinc, however, emulsifiers also can be present as well as minor amounts of polyunsaturated fatty acids in particular DHA.

Preferably, the food product or health supplement contains sufficient levels of flavonol glycosides to allow a daily intake equivalent to at least 0.1mg quercetin aglycon per kg of bodyweight.

The application will now be described with reference to the following non-limiting examples.

EXAMPLES

OVERVIEW OF PROTOCOL

The effects of a tomato paste, enriched with quercetin-glycosides (mainly rutin and isoquercitrin), on blood pressure were examined on Spontaneously Hypertensive Rats (SHR). SHR are extensively used in research to assess the effects of bioactive agents on blood pressure.

The SHR were equipped with a blood pressure measuring telemetry device that allows blood pressure to be continuously and non-invasively monitored. Flavonol enriched tomato paste in different forms was administered via the diet of the rats.

The effects of this diet (flavanol enriched tomato paste) was compared with a control tomato paste (contains a low level of flavonols), a diet containing pure quercetin aglycon and a flavonoid-free diet.

Blood pressure was measured for a period of 10 seconds every 5 min over 24 hours for 3 consecutive days per week using a telemetry device. Each diet was administered for a period of 5 weeks.

SCREENING OF TOMATO PASTES TO DETERMINE FLAVONOL LEVELS

A rapid screening method was required for the differentiation of high and low flavonoid tomato pastes to be used in the rat clinical trial. Rutin (quercetin-3-rutinoside) and Isoquercitrin (quercetin-3-glucoside) are the primary flavonoids (flavonol glycosides) of interest with rutin being the major flavonoid glycoside expected in the high flavonoid pastes. Both thin layer chromatography procedures and UV spectrophotometric procedures can be used as quality control procedures. Flavonoids (both aglycone and flavonol glycosides) typically show characteristic absorbancies at 270nm and 370nm.

MATERIALS

Mineralight UV Lamp UVGL 58, 254/365nm

Pierce Reacti-Therm/Reacti-Vap Heated Block 18790 with 18780 Evaporation Unit.

Screw capped glass vials, Fisher, TUL-520-060D, 14mL, neutral glass.

Methanol and Ethanol (Analytical Grade reagents minimum quality) ex Fisher.

Rutin, 95%, ex Sigma, (also Isoquercitrin, HPLC grade, if required)

Centrifuge

Literature: Plant Drug Analysis: A Thin Layer Chromatography Atlas by H. Wagner and S. Bladt

www.machery-nagel.com as TLC00003 TLC Separation of Flavonoids (DAB) Machery-Nagel Catalogue Edition 3, page 267.

SAMPLE EXTRACTION

A sample of low flavonoid paste and a sample of high flavonoid paste 1g +/- 0.1g of paste was put into a 15mL screw capped glass vial. 10mL of methanol was added and the mixture was vibromixed thoroughly for 1 minute. The tube was heat sealed on a Pierce Reacti-Therm (or equivalent) heated block for 30 minutes at 60°C, vibromixing the mixture every 5-10 minutes, preferably within a fume cupboard. The mixture was allowed to cool. If

required the sample tubes were centrifuged on a bench top centrifuge (swing-out) for 10 minutes at 2000rpm. The clear upper methanol layer was pipetted off into a clean glass vial. The methanol was filtered through a 0.2uM PTFE syringe filter to remove both lycopene and any residual solid material.

SCREENING BY UV SPECTROPHOTOMETRY

Methanol extracts characterised under UV light can be more quantitatively characterised by UV Spectrophotometry. The solutions were diluted typically by a factor of 10:1 and their UV absorbance spectra determined 190nm-600nm.

Using cuvettes suitable for UV measurements down to 190nm, methanol was placed in both the reference and sample positions in the UV spectrometer. The "blank" solvent background spectrum was checked and established. Cuvettes used for sample analysis required thorough rinsing with methanol and drying with tissue and under nitrogen between measurements. The spectra ranging 190nm -600nm of an approximately 0.01mg/mL solution of rutin standard in methanol was measured. The spectral maxima typically observed 260nm-270nm and 360nm-370nm was recorded. The spectrum and absorbance maxima obtained for rutin are shown in Figure 7 and Table 1. The control paste and high flavonoid paste were analysed as methanol extract solutions by determining the spectra ranging 190nm. The absorbance was

recorded at the observed spectral maxima for each sample typically 260nm-270nm and 360nm-370nm as shown in Figure 8 and Table 2.

Table 1. Absorbance Maxima Measured For Rutin Standard (0.1mg/mL)

Sample	Absorbance	
	Wavelength	
	359.0nm	258.5nm
Rutin standard	0.28	0.32

Table 2. Absorbancies For Control And C11+ High Flavonoid Tomato Pastes (Literature Maxima of 270nm and 370nm)

Sample	Absorbance	
	Wavelength	
	370.0nm	270.0nm
Control Paste	0.1406	1.1006
C11+ High Flavonoid	0.4368	1.3793

<u>Protocol for SHR Testing</u>

Design of the study

24 SHR were used in the study and they were equipped with a blood pressure measuring telemetry device to be continuously and non-invasively monitored. Four diets were given in an incomplete block design of 2 x 5 weeks intervention. All rats had an acclimatisation period (week 0), feeding training

and reversal of circadian rhythm (week 1), implantation of transmitter and a recovery period (weeks 2 and 3) and a run-in period (week 4) prior to the interventions

Test system using Male, Spontaneously Hypertensive Rats (SHR)

The age of the rats at the beginning of the first intervention ranged from 11 to 16 weeks. The animals were marked with their animal number by means of an earmark. Throughout the study the animals were housed individually in reversed 12 hrs light/12 hours dark-cycle 9 from 8.00 – till 8.00 with free access to drinking water.

Test article

The test substance in this study was given via the diet. The diets were given according to an incomplete block design, which means that every rat received 2 of the 4 diets. Water was supplied ad libitum 24-hours a day. The experimental diets (diet B, BQ, T and TQ) were given between 8.00 - 9.00 AM (just before start of the dark period). The amount of experimental diet was adjusted to the mean weight of the rats which receive the experimental diet, approximately 2.5g porridge or paste /100g body weight. After the experimental diet all rats received ad libitum the flavonoid-free semi-synthetic diet (diet B) from 9.00 AM - 4.00 PM. If some of the experimental diet was left it was mixed thoroughly with the upper part of the flavonoid-free semi-

synthetic diet. From 4.00 PM until 8.00 AM the following day the rats received no food.

The following test diets were used in the study:

Diet B - flavonoid-free semi-synthetic diet. The composition of this diet is given in Table 3.

Portions of the semi-synthetic diets were prepared prior to each 4 or 5-week feeding period and stored at –20°C in aliquots suitable for one day of feeding. These aliquots were thawed and mixed appropriately with water prior to use.

TABLE 3 : Flavonoid-free semi-synthetic diet :

Total diet composition : Diet B

Ingredient	grams per/kg	en%	kJ
Calcium-caseinate (15.7 kJ/g)	150.5	16	2357.7
Vitamin-mixture	10.7		
Mineral-mixture	36.7		
Arbocel BC-200	52.5		
Fat blend (37.7 kJ/g)*	78.3	20	2947.1
Choline Bitartrate	2.6		
L-cysteine Hydrochloride	1.9		
Maize starch (13.7 kJ/g)	667.0	64	9430.6
Total	**1000**	**100**	**14735.4 /kJ**

***) Composition of the fat blend: SAFA:MUFA:PUFA = 1:1:1**

- **Coconut oil 5.00 grams**
- **Hozol 2.46 grams**
- **Lard 49.02 grams**
- **Palm oil 1.00 gram**
- **Sunflower 42.53 grams**

Vitamin mix

Ingredient	**g/kg mix**
Nicotinamide	3.00
Ca^{++}pantothenate	1.60
Pyridoxine B6	0.70
Thiamine monitr. B2	0.60
Riboflavine B1	0.60
Folic acid	0.20
Biotin	0.02
Vitamin B12	5.00
Vitamin E (50%)	15.00
Vitamin A,500000 IE	0.80
Vitamin D_3	1.00
Vitamin K_1 (phylloq)	0.10
Maize starch	971.38
Total	**1000.00**

<u>Mineral mix</u>

Ingredient		g/kg mix
Calcium Carbonate	$CaCO_3$	236.91
Potassium-dihydro phospate	KH_2PO_4	196.00
Sodium chloride	NaCl	74.00
Magnesium oxide	MgO	24.00
Potassium citrate	$C_6H_5K_3O_7.H_2O$	70.78
Potassium sulphate	K_2SO_4.	46.60
AIN mineral mix*		91.71
Maize starch		260.00
Total		**1000.0**

AIN mineral mix*

Ingredient		g/100g mix
Potassium chromium(III)sulphate	CrK(SO4).12H2O	0.2750
Cupper carbonate	CuCO3.Cu(OH)2	0.3000
Sodium fluoride	NaF	0.0635
Potassium iodate	KIO3	0.0100
Iron-citrate	C6H5FeO7.5H2O	6.0600
Manganese carbonate	MnO3	0.6300
Sodium selenite	Na2SeO3	0.0154
Zinc carbonate	ZnCO3.2Zn(OH)2.H2O	1.6500
Sodium molybdate	Na2MoO4.2H2O	0.0110
Sodium meta-silicate	Na2SiO3	1.4500
Litium Chloride	LiCl	0.0174
Boronic acid	H3BO3	0.0815
Nickel carbonate	2NiCO3.3Ni(OH)2.4H2O	0.0318
Ammoniumvanadate	NH4VO	0.0066
Maize starch		81.1080
	Total	**91.71**

For the experimental diet 1g powder was mixed with 1.5 ml water per 100g body weight of the rat. For the diet between 9.00 AM - 4.00 PM the powder was mixed 1:1, approximately 40 g porridge per rat was given. The porridge was freshly made every morning.

Diet BQ - semi-synthetic diet containing pure quercetin aglycone

This diet consists of the flavonoid-free semi-synthetic diet containing 3g quercetin / kg powder diet B. The powder was used directly after thawing and was mixed with water, i.e. 1g powder + 1.5 ml water per 100g body weight of the rat. The diet was freshly made every morning.

Diet T - normal tomato paste (not enriched)

Normal tomato paste was used which contained an equivalent of 1.73 mg flavonoids/100 g wet weight paste (as determined by HPLC analysis). Immediately before the paste was used the flavonol level was qualitatively checked by UV spectroscopy. The amount of tomato paste given to the rat every day was 2.5g / 100g BW.

Diet TQ - tomato paste from genetically modified tomatoes

The tomato paste from genetically modified tomatoes made in accordance with WO 00/04175 which contained the equivalent of 48.8 mg quercetin aglycone per 100 g wet weight paste was used (level determined by HPLC analysis). A qualitative check was carried out immediately before use as described above (T-diet). As expected all tomato paste from genetically modified tomato samples contained high flavonoid levels. The amount of tomato paste given to the rat every day was 2.5g / 100g BW.

Results: Laboratory analysis

Determination of blood pressure (telemetry)

Blood pressure was measured every 5 minutes for 10 seconds for 3 consecutive days per week and per rat. The different parameters, i.e. systolic, mean, diastolic pressure, were calculated. The mean of a specific parameter for a specific period of a day per rat was calculated. The value per week was calculated as the mean value of the 3 separate days. The results per rat are expressed as an increase or decrease of the specific parameter compared to week 0 (mmHg).

The specific time periods were:

9 AM – 7 AM (22 hrs)

5 AM – 7 AM (early morning period)

In the analysis, the results of the 1st and 2nd intervention periods were combined. The week numbers used in the analysis is as follows:

wk 0 = week before intervention (run-in + last week of wash-out)

wk 1 - 5 = 1st – 5th intervention weeks

In this way 41 rats were analysed. This should have been 48 but because of transmitter-tip 'silting' a loss of a number of signals occurred.

Paste samples

Immediately after opening each can of paste (GM and control), a 5-10g sample of the paste was taken and stored at -20°C for future flavonol analysis by HPLC. In addition, a small sample of the paste was used on the day of diet preparation and prior to administration, for crude analysis by UV spectrometry to confirm whether the paste had low or high levels of flavonols.

RESULTS

The results shown in figures 1 to 6 clearly demonstrate a link between the reduction of hypertension and the administration of tomato paste which exhibits high flavonol levels.

Systolic blood pressure and diastolic blood pressure are indicators or risk factors of cardiovascular events in later life (Safar M.E. "Epidemiological Findings Imply That Goals for Drug Treatment of Hypertension Need to be Revised" (2001) Circulation 103:1188-1190). Systolic blood pressure is believed to be a good measure of hypertension.

There is a consistent trend for a time dependent increase in blood pressure of the SHRs. As shown in the figures, the only diet that seems to be able to counteract this increase is the diet containing high levels of flavonol glycosides (TQ). This is most notable for the diastolic blood pressure during

the early morning period, where after 5 weeks the group fed a basic diet lacking flavonols shows an increase in average diastolic blood pressure of 8 mm Hg relative to week 0, whereas the group fed the TQ diet shows an average decrease of 3 mm Hg relative to week 0. The resulting difference in average diastolic blood pressure between those two groups, after 5 weeks, is therefore approx 11 mm Hg. The differences in average mean blood pressure show the same trend. The differences in average systolic blood pressure are smaller but still follow the same trend. Given that relatively modest reduction in blood pressure can lead to substantial decreases of cardiovascular risk factors, the observed effects are significant. Moreover these results raise the possibility of providing food products with blood pressure lowering effects. Consequently, people at risk of becoming hypertensive may delay this possibility without taking medication.

Interestingly, the SHRs fed ordinary tomato paste exhibited a slower rate of time dependent blood pressure increase, compared to the B and BQ fed rats. This could possibly be explained by the fact that ordinary tomato paste contains low levels of flavonol-glycosides.

As noted earlier, even small blood pressure decreases contribute to reductions of cardiovascular and all-cause mortality. This is true for systolic and diastolic blood pressure. In addition to the figures for systolic blood pressure, the figures for diastolic blood pressure point into the same direction.

In an analysis of the Framingham Heart Study experience, Cook et al "Implications of small reductions in diastolic blood pressure for primary intervention" (1995) Arch. Int. Med. 155: 701-709 reported that a 2-mm Hg reduction of diastolic blood pressure for white US residents 35 to 64 years of age would result in a 17% decrease in the prevalence of hypertension, a 14% decrease in the risk of stroke and transient ischemic attacks, and a 6% reduction of coronary heart disease. Given the fact that the risk for cardiovascular disease is higher in people with higher blood pressure, the benefits of small blood pressure reductions among these people will be higher than the population-based numbers cited above. The present invention, therefore, shows that important risk reductions can be attained with a food containing or enriched in flavonol glycosides.

CLAIMS

1. Use of a plant or plant extract which exhibits naturally high, altered or increased levels of flavonol glycosides in the reduction of hypertension

2. Use according to claim 1 wherein the plant or plant extract provides a daily dosage of flavonol glycoside equivalent to from about 0.1 to about 20mg of flavonol aglycon per kg of body weight.

3. Use according to claim 1 or 2 wherein the plant is preferably a vegetable or fruit.

4. Use according to any of claims 1 to 3 wherein the plant is a tomato, onion, apple, blueberry, broccoli, tea, buckwheat.

5. Use according to any of claims 1 to 4 wherein the plant is genetically modified to exhibit altered levels of flavonol glycosides.

6. Use according to claim 5 wherein the genetically modified plant comprises two or more transgenes each encoding a different transcription factor for flavonoid biosynthsis or a sequence functionally equivalent thereto, stably

incorporated into its genome such that its ability to produce flavonoids other than anthocyanins is altered.

7. Use according to claim 6 wherein the genetically modified plant comprises a DNA sequence encoding the maize C1 transcription factor in combination with a DNA sequence encoding the maize Lc transcription factor.

8. Use according to claim 5 wherein the plant is genetically modified with the chalcone isomerase (CHI) gene, preferably under the control of CaMV 35S promoter.

9. Use according to claim 5 wherein the genetically modified plant expresses chalcone synthase and flavanol synthase simultaneously and optionally expresses chalcone isomerase and/or flavonone-3-hydroxylase.

10. Use according to any of the preceding claims wherein the flavonol glycoside is a quercetin glycoside, preferably rutin, isoquercitrin or both.

11. Use according to any of the preceding claims wherein the genetically modified vegetable or fruit is in the form of a vegetable or fruit extract.

12. Use according to any of claims 1 to 10 wherein plant or plant extract is in the form of a paste or other processed form.

13. Use according to any of the preceding claims wherein the vegetable or fruit is a tomato.

14. A food product containing a plant or plant extract, preferably a vegetable or fruit or vegetable or fruit extract, which exhibits naturally high, increased or altered levels of flavonol glycosides wherein the food product is selected from the group consisting of spreads, margarines, creams, mayonnaises, ice creams, fillings, confectioneries, health bars, cereals and health drinks and tea.

15. A health supplement containing a plant or plant extract, preferably a fruit or vegetable or fruit or vegetable extract, which exhibits naturally high, increased or altered levels of flavonol glycosides.

16. A food product according to claim 14 or 15 wherein the plant is genetically modified.

17. A food product or health supplement according to claim 14, 15 or 16 which provides a daily intake equivalent to at least 0.1 mg quercetin aglycon er kg of bodyweight.

18. A food product or health supplement comprising a plant or plant extract, which has been genetically modified in accordance with any of claims 5 to 9.

19. A method for the treatment of hypertension in mammals by administering to the mammal an effective amount of a plant or plant extract which may be genetically modified, preferably a fruit or vegetable which exhibits naturally high, increased or altered levels of flavonol glycosides in the reduction of hypertension, or a food product or health supplement according to any of claims 14 to 18.

ABSTRACT

PLANT AND PLANT EXTRACTS AND THEIR USE

The present invention relates to use of a plant or plant extract, preferably a vegetable or fruit, exhibiting naturally high, altered or increased levels of flavonol glycosides, in the reduction of hypertension in mammals, such as man, and novel products containing those flavonol glycosides.

Figure 1a

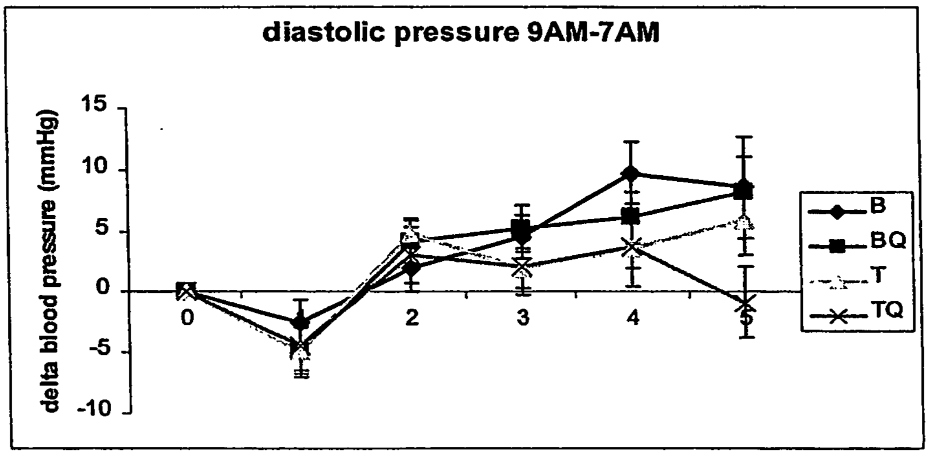


Figure 1b

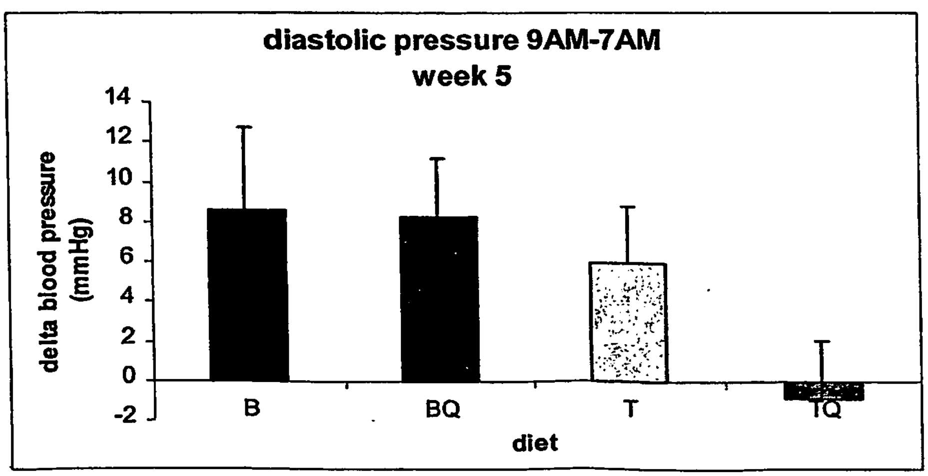


Figure 2a

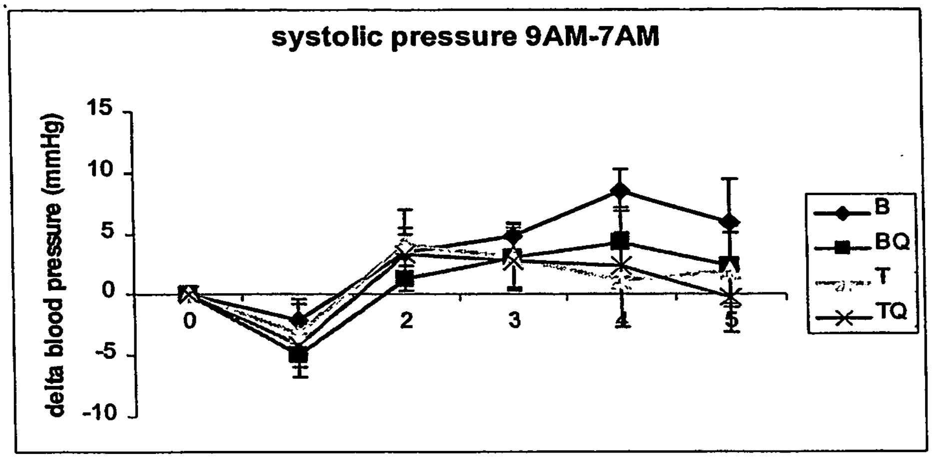


Figure 2b

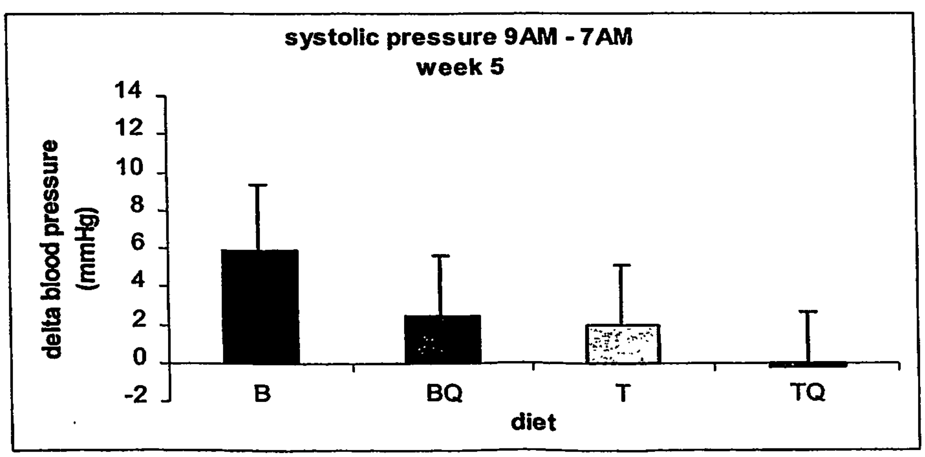


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Figure 3a

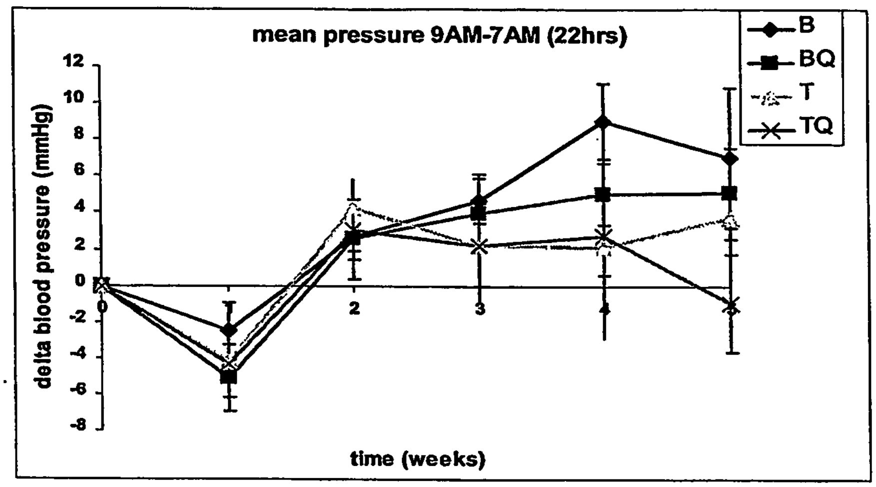


Figure 3b

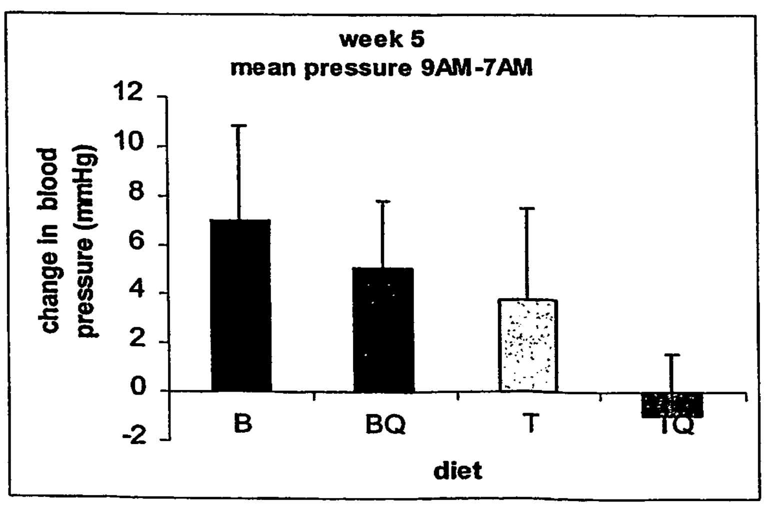


Figure 4a

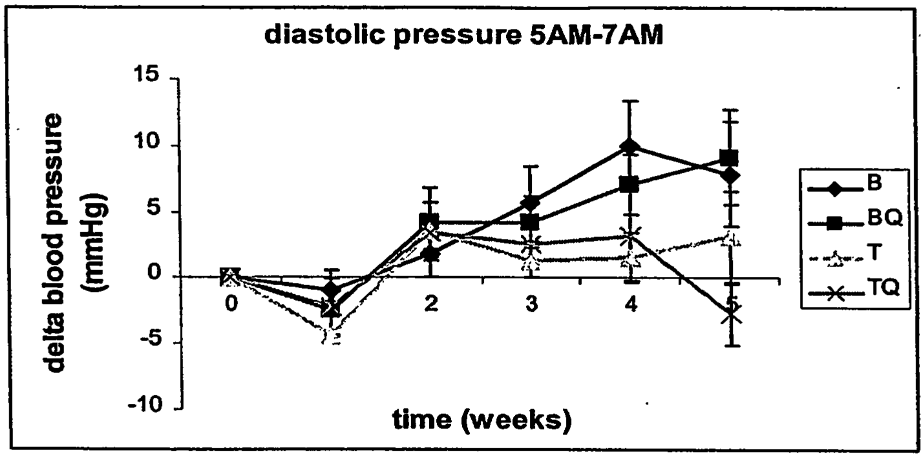


Figure 4b

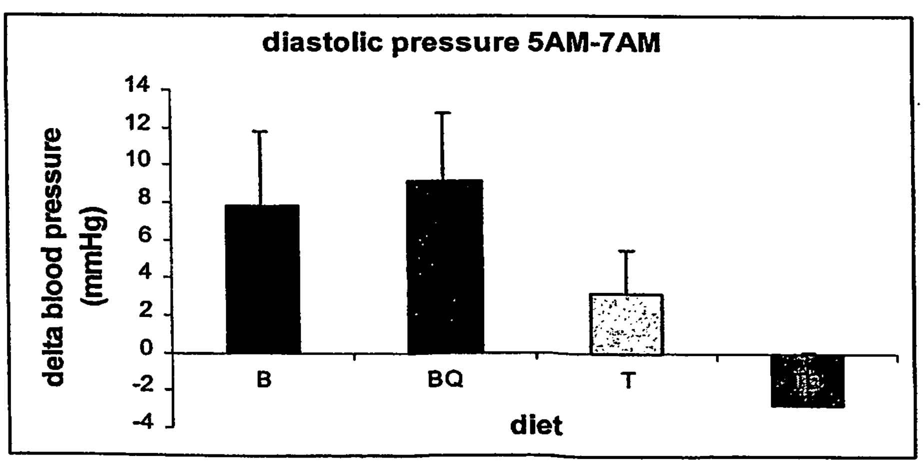


Figure 5a

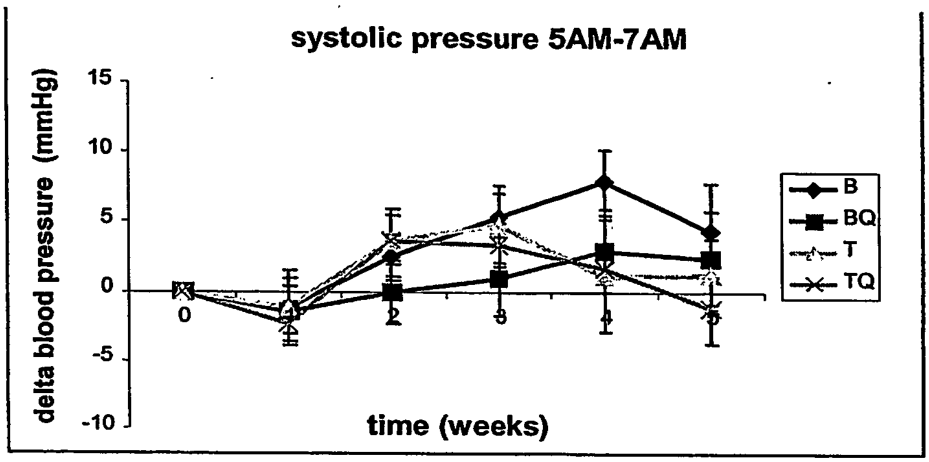


Figure 5b

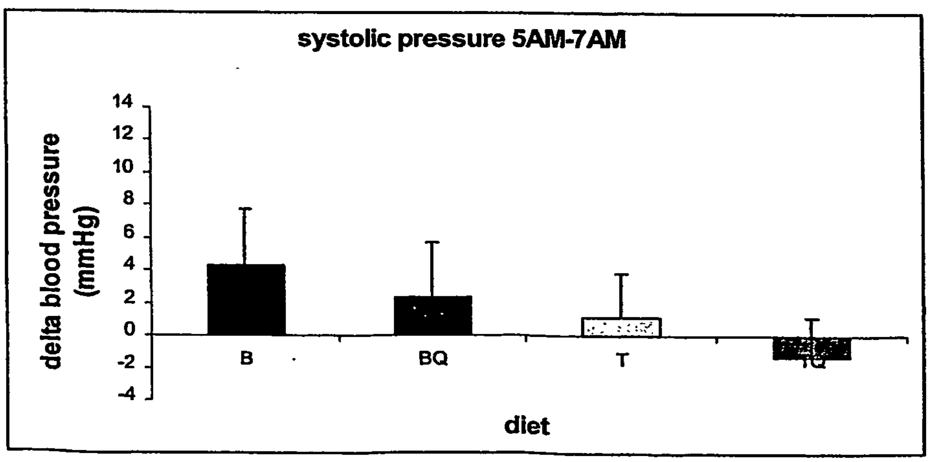


Figure 6a

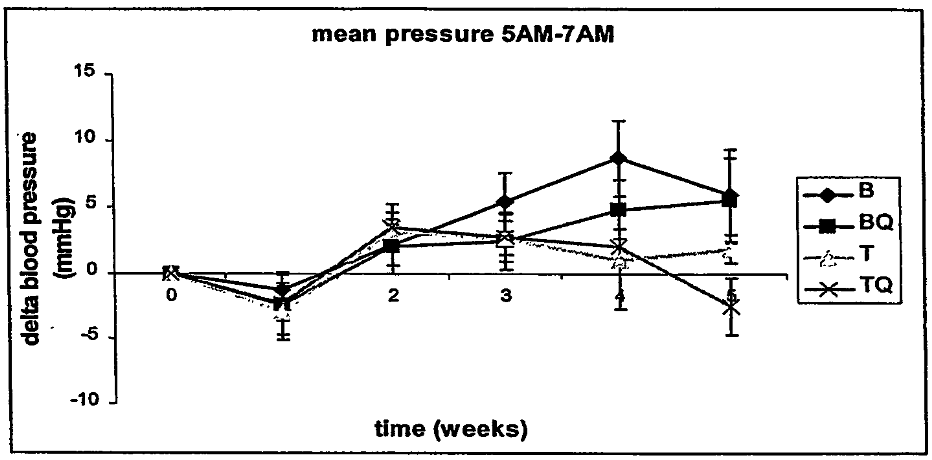


Figure 6b

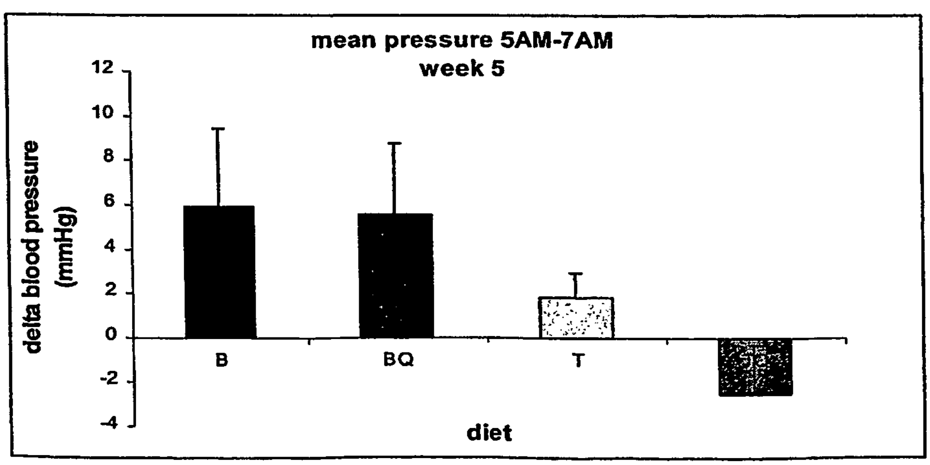
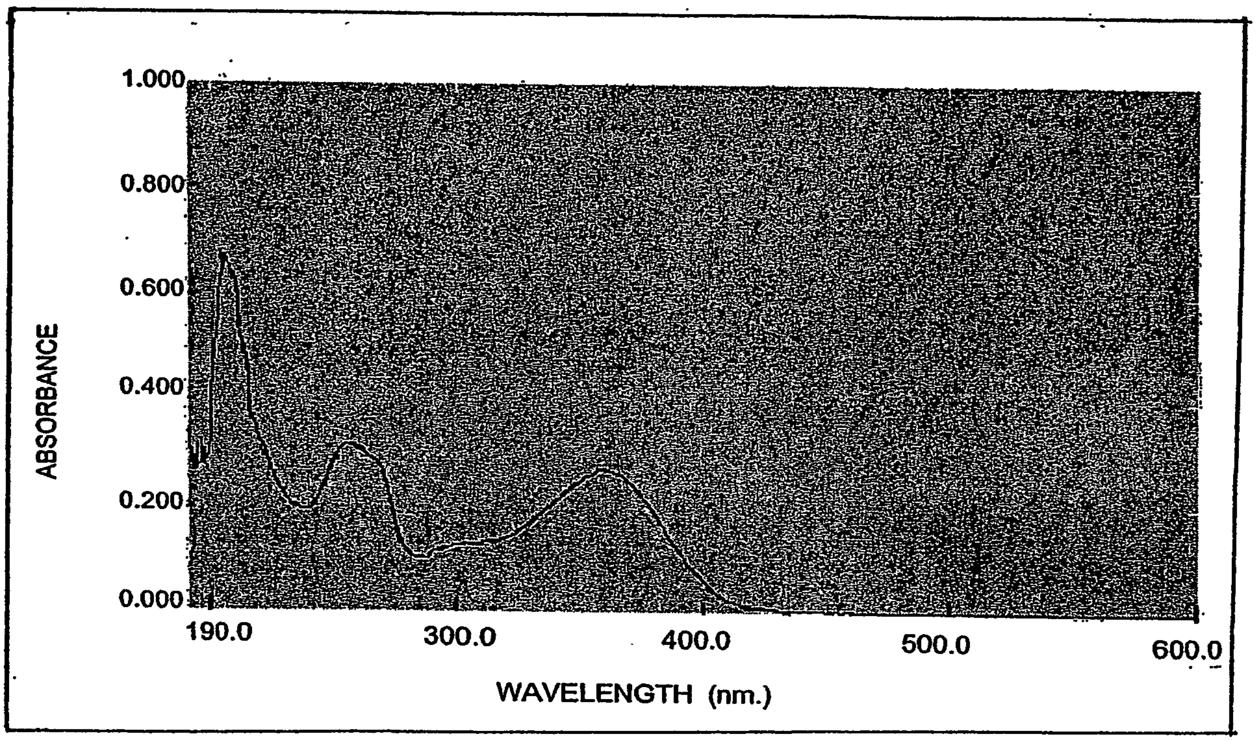


Figure 7


1.000
0.800
0.600
0.400
0.200
0.000
ABSORBANCE
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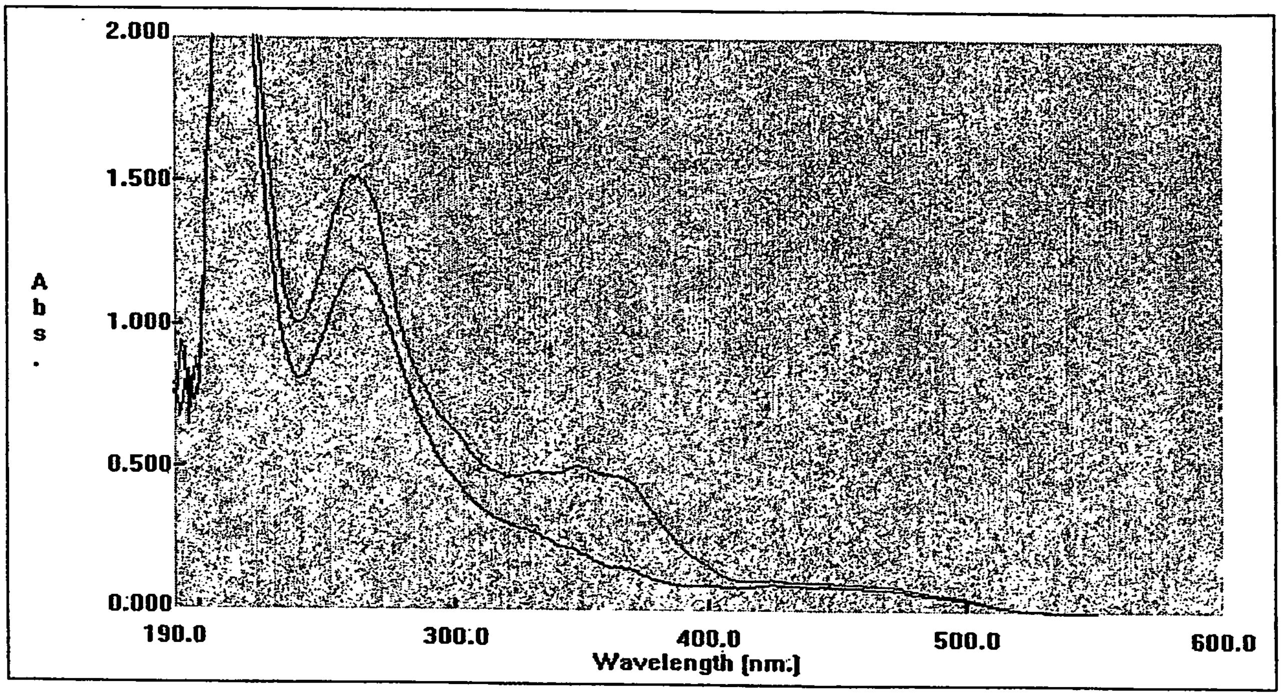
300.0
400.0
500.0
600.0
WAVELENGTH (nm.)

Figure 8


2.000
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